APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1992, p. 832–839
0099-2240/92/030832-08$02.00/0

Vol. 58, No. 3

Cloning, Sequence, and Phenotypic Expression of *katA*, Which Encodes the Catalase of *Lactobacillus sake* LTH677

HERMANN J. KNAUF, RUDI F. VOGEL,* AND WALTER P. HAMMES

Institut für Lebensmitteltechnologie, Universität Hohenheim, Garbenstrasse 25, 7000 Stuttgart 70, Germany

Received 26 July 1991/Accepted 17 December 1991

***Lactobacillus sake* LTH677 is a strain, isolated from fermented sausage, which forms a heme-dependent catalase. This rare property is highly desirable in sausage fermentation, as it prevents rancidity and discoloration caused by hydrogen peroxide. A gene bank containing *Mbo*I fragments of chromosomal DNA from *Lactobacillus sake* LTH677 in *Escherichia coli* plasmid pBR328 was constructed. The catalase gene was cloned by heterologous complementation of the Kat$^-$ phenotype of *E. coli* UM2. The catalase structural gene, designated *katA*, was assigned to a 2.3-kb region by deletion analysis of the originally cloned fragment in plasmid pHK1000. The original chromosomal arrangement was determined by Southern hybridization. Protein analysis revealed that the catalase subunit has a molecular size of 65,000 Da and that the active catalase possesses a hexameric structure. The molecular size of the subunit deduced from the nucleotide sequence was determined to 54,504 Da. The N-terminal amino acid sequence of the 65,000-Da protein corresponded to the one deduced from the DNA sequence. After recloning of *katA* in the *E. coli-Lactococcus* shuttle vector pGKV210, the gene was successfully transferred and phenotypically expressed in *Lactobacillus casei*, which is naturally deficient in catalase activity.**

Lactobacilli are widely used in food fermentation. Their main contribution to the fermentation process is acidification, which prevents growth of food poisoning bacteria and is the prerequisite for coagulation of milk in the production of cheese and yogurt or coagulation of soluble meat proteins to form a firm matrix during sausage fermentation. During fermentation, hydrogen peroxide may be formed and accumulated, severely decreasing the sensory quality of the product. For example, in meat products the accumulation of hydrogen peroxide can lead to rancidity and discoloration (24). Therefore, catalase activity is a desirable property for starter cultures used in food fermentations.

Lactobacilli are commonly regarded as devoid of catalase activity, but there are several reports of strains of lactobacilli which exhibit catalase activity if exogenous heme, which is naturally present in meat products, is provided. Wolf and Hammes (34) showed that these organisms possess the ability to form the catalase apoenzyme and can incorporate the heme compound to form an active enzyme. Cloning and analysis of the structural gene encoding catalase could indicate whether in some lactobacilli this property was preserved during evolution or was a property newly acquired by some species. Furthermore, the catalase gene could be transferred to and expressed in other lactobacilli used in food fermentation to improve the sensory quality of the products. It also may be used as a food grade marker gene in catalase-deficient lactic acid bacteria. In this report, we provide data on the cloning of the catalase structural gene of *Lactobacillus sake* LTH677 and its expression in *Escherichia coli* and *Lactobacillus casei*. DNA homology of the catalase gene to the *E. coli* catalase (hydroperoxidase) HPI gene and catalase genes of other lactic acid bacteria was investigated.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *E. coli* and lactobacilli were grown in LB medium (6) and MRS medium (7), respectively, as described previously (10). For detection of catalase activity in lactobacilli, a solution containing hematin (5 mg/ml) in 0.2 M KOH was added to the medium to a final concentration of 30 mM. Selective media contained ampicillin (50 μg/ml) or erythromycin (100 μg/ml or 10 μg/ml for *E. coli* or lactobacilli, respectively).

DNA techniques. Plasmid DNA of *E. coli* or lactobacilli was isolated according to the method of Birnboim and Doly (2) or Anderson and McKay (1), respectively. Chromosomal DNA of *L. sake* LTH677 was isolated as described before (16). DNA was further purified by cesium chloride density gradient centrifugation (25). Restriction enzymes and ligase were purchased from GIBCO-BRL GmbH (Eggenstein, Germany) and used according to the instructions of the supplier. DNA fragments were analyzed in agarose gels (25). *Hin*dIII fragments of bacteriophage λ DNA (Boehringer GmbH, Mannheim, Germany) were used as molecular weight markers.

Southern hybridization was performed as described by Sambrook et al. (25) and included a depurination step (33). DNA probes were labeled with digoxigenin-11-dUTP according to specifications provided with the nonradioactive labeling and detection kit (Boehringer). Prehybridization, hybridization, and blocking were performed in the presence of 3× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate, pH 7.0) at 58°C. Different stringencies were applied by altering the salt concentrations and temperature in the washing procedure. Selective hybridization of sequences sharing 100% or more than 60% homology was achieved by washing the filters in 0.16× SSC at 68°C or in 3× SSC at 58°C. Digoxigenin-labeled λ DNA was added to the probe.

DNA sequencing was performed essentially as described by Sanger et al. (26) with a T7 sequencing kit (Pharmacia LKB, Freiburg, Germany). Reactions were performed as

TABLE 1. Bacterial strains and plasmids used in this study

Bacteria and plasmids	Relevant properties	Source or reference
Lactobacillus sake LTH667	Kat^+	Isolate from fermented sausage
L. sake LTH682	Kat^+	Isolate from fermented sausage
L. casei subsp. *casei* 102S	Plasmid-free derivative of the type strain ATCC 393, deficient in catalase activity, highly transformable by electroporation	3
L. pentosus DSM20314	Kat^+	Type strain
Pediococcus acidilactici DSM20286	Kat^+	Type strain
Escherichia coli UM2	*katE2 katG15*	20
E. coli BHB2600	803 *supE*$^+$ *supF*$^+$ r_K^- m_K^+ *met*	15
pBR328	Amp^r Tet^r Cm^r	28
pUC19	Amp^r	36
pBT22	Amp^r Tet^s *katG*$^+$	30
pGKV210	Em^r *E. coli-Lactococcus* shuttle vector	31
pHK1000	Amp^r Tet^s Cm^r	This work
pHK1100	Amp^r Tet^s Cm^r	This work
pHK1200	Amp^r Tet^s Cm^s	This work
pHK1300	Amp^r	This work
pHK1400	Amp^r	This work
pHK1150	Amp^r	This work
pHK1250	Amp^r	This work
pHK1155	Em^r	This work

described in the manufacturer's manual. Subclones were constructed in pUC19, and the insert was sequenced from both sides by using the universal or reverse oligonucleotide primers. When necessary, synthetic oligonucleotides deduced from the sequence were constructed and employed as primers to obtain overlapping sequences. DNA sequences and amino acid sequences were analyzed with a microcomputer system and DNAsis software (Pharmacia LKB).

Transformation of *E. coli* and *L. casei*. *E. coli* cells were transformed essentially by the method of Hanahan (12) or by electroporation using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described by Dower et al. (8). *L. casei* was transformed by electroporation as described by Chassy and Flickinger (3).

Construction and amplification of the genomic library. Chromosomal DNA of *L. sake* LTH677 was partially cleaved with *Mbo*I. Fragments of approximately 4 kb in length were isolated from preparative gels by the method of Dretzen et al. (9). The fragments were ligated into the *Bam*HI-cleaved plasmid pBR328. The ligation mix was used to transform *E. coli* BHB2600. After being cultured, the transformants were washed off the agar plates and collected by centrifugation. The cells were resuspended in a total volume of 1 ml of growth medium and mixed with an equal volume of sterile glycerol. The cells containing the gene library were stored at −30°C. For amplification of the gene library, LB broth (500 ml) was inoculated with 5 ml of an overnight culture (5 ml of LB broth inoculated with 50 μl of storage culture) and incubated for 8 h. Cells were collected by centrifugation, and plasmid DNA was isolated.

Catalase activity test. Catalase-positive *E. coli* clones were screened by flooding the colonies on agar plates with hydrogen peroxide (0.87 M) and immediately picking with a sterile toothpick those that produced oxygen bubbles. Catalase-positive *L. casei* transformants were detected as oxygen-producing clones after being picked and submerged in hydrogen peroxide (0.87 M) with a sterile toothpick. A quantitative analysis of catalase activity was performed according to the method of Sinha (27) with some modifications.

Exponentially growing cells (5 ml) were harvested by centrifugation and resuspended in phosphate buffer (0.1 M, pH 7.0) to obtain a concentration of 1.0×10^8 cells per ml. Cell suspensions (3 ml) were mixed with phosphate buffer (3 ml, 0.1 M, pH 7.0) and H_2O_2 (4 ml, 0.2 M). Immediately 0.5 ml was withdrawn and added to 1 ml of a solution containing 0.33 ml of dipotassium chromate (50.0 g/liter) and 0.67 ml of glacial acetic acid. A sample was withdrawn every minute in the same way for 10 min. The samples were boiled for 10 min, and the developing green color was measured photometrically at 570 nm. A decrease in extinction of 0.1 corresponded to a decrease in H_2O_2 of 150 μmol. Catalase activity was determined as the decrease in H_2O_2 concentration per minute per 3×10^8 CFU.

Protein pattern analysis. Analysis of soluble proteins was performed according to the method of Moore et al. (21). An overnight culture of *E. coli* or *Lactobacillus* cells (5 or 10 ml, respectively) was harvested by centrifugation. After being washed in Tris-HCl buffer (0.15 M, pH 7.0), the cells were resuspended in 150 μl of Tris buffer and transferred to 5-ml screw-cap tubes containing 150 mg of glass beads 0.5 mm in diameter. Cell lysates were obtained by shaking the tubes in a cell mill (Bühler, Tübingen, Germany) at a frequency of 2,000 vibrations per min for 3 min at 0°C. After centrifugation, the proteins in 20 μl of the supernatant were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels (17). Standard protein markers (GIBCO-BRL) were used as molecular weight markers.

Activity staining of the catalase. Total soluble proteins were isolated as described above and separated on a 7.5% nondenaturing polyacrylamide gel. The gel was stained by the method of Clare et al. (5) using 5 mM H_2O_2.

Native-molecular-weight analysis. For native-molecular-weight analysis, the method of Hedrick and Smith (14) was used. In this method, crude extracts were electrophoresed on a series of gels of various acrylamide concentrations from 5 to 11%. The R_f of the protein relative to the front of the bromphenol blue tracking dye was determined for each gel after staining for catalase activity as described above. The retardation coefficient for each protein was determined from

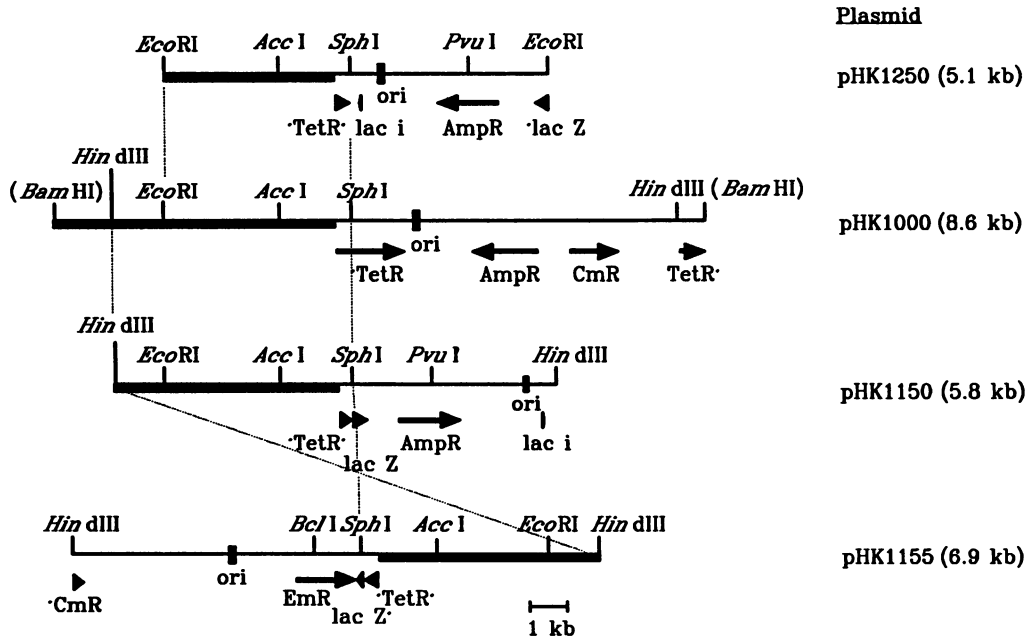

FIG. 1. Construction of different plasmids harboring the catalase gene. pHK1250, *Eco*RI-*Sph*I fragment of pHK1000 cloned into pUC19; pHK1150, *Hin*dIII-*Sph*I fragment of pHK1000 cloned into pUC19; pHK1155, *Hin*dIII-*Sph*I fragment of pHK1150 cloned into pGKV210. ori indicates the origin of replication. Also indicated are regions encoding resistance to tetracycline (TetR), ampicillin (AmpR), chloramphenicol (CmR), and erythromycin (EmR). Thick lines indicate *L. sake* DNA; thin lines indicate vector DNA. Arrows and arrowheads indicate directions of transcription.

the slope of a graph of 100× log (100 × R_f) against the polyacrylamide concentration. The retardation coefficient was then plotted against the logarithm of the molecular size of the protein. A series of proteins with known molecular sizes of 67,000 to 669,000 Da were obtained from Pharmacia and run as standards. For the standards, the gels were stained with Coomassie brilliant blue dye.

Subunit analysis. Proteins exhibiting catalase activity were analyzed in a two step electrophoresis procedure. Proteins exhibiting catalase activity were detected as negatively stained (light) areas in nondenaturing gels. Corresponding areas were cut out from unstained nondenaturing gels run in parallel. These gel pieces were embedded in a collecting gel of a denaturing SDS-polyacrylamide gel. Proteins trapped in the gel pieces were detected after separation on a 7.5% polyacrylamide denaturing gel and staining for protein with Coomassie brilliant blue dye.

Protein sequence determination. Total soluble proteins were separated on an SDS–7.5% polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Eschborn, Germany) and stained with Coomassie brilliant blue dye. The band corresponding to catalase subunits was cut out. The amino acid sequence of the N terminus of the filter-bound protein was determined by gas-phase microsequencing in a model 471A protein sequencer (Applied Biosystems GmbH, Weiterstadt, Germany) according to the instructions of the supplier.

Nucleotide sequence accession number. The GenBank accession number for the *katA* gene is M84015.

RESULTS

Cloning of the catalase gene. The gene library consisted of about 10,000 independent clones with genomic inserts of approximately 4 kb. After amplification, plasmid DNA was transferred by electroporation in *E. coli* UM2. One clone that exhibited catalase activity was isolated. The clone harbored a plasmid of 8.6 kb referred to as pHK1000 and carried a 3.7-kb insert of *L. sake* DNA.

Location of the catalase gene in pHK1000. Figure 1 shows a restriction map of pHK1000. No digestion was observed with *Bcl*I, *Nco*I, *Bam*HI, *Cla*I or *Pvu*I. Several deletion derivatives of pHK1000 were constructed and analyzed for their capacity to complement the Kat$^-$ phenotype of *E. coli* UM2. The results are summarized in Fig. 2. The catalase structural gene was assigned to a 2.3-kb region located between the *Eco*RI site and the right-end *Mbo*I cloning site in pHK1000. Further deletions resulted in formation of plasmids not capable of complementation of *E. coli* UM2.

In order to investigate whether the *Lactobacillus* promoter had been cloned, the catalase gene was cloned into pUC19 in two different orientations. The construction of the two resulting plasmids, pHK1150 and pHK1250, is depicted in Fig. 1. Both plasmids complemented the Kat$^-$ phenotype of *E. coli* UM2. This indicates that sequences recognized as a promoter by *E. coli* are present on the cloned fragment.

Location of the catalase gene in the chromosome of *L. sake* LTH677. The presence of *L. sake* LTH677 genomic DNA in pHK1000 was confirmed by Southern hybridization with biotinylated pHK1000 as a probe. The vector pBR328 did not produce a signal. A restriction map of the region in the chromosome surrounding the catalase gene was constructed on the basis of Southern hybridizations. For this purpose, the 1.7-kb *Acc*I fragment inside the putative coding region was used as a probe for *L. sake* LTH677 DNA, which was cut in several single and double restriction enzyme digestions.

Representative hybridization patterns are depicted in Fig. 3. The mapping of the catalase gene on pHK1000 and on the

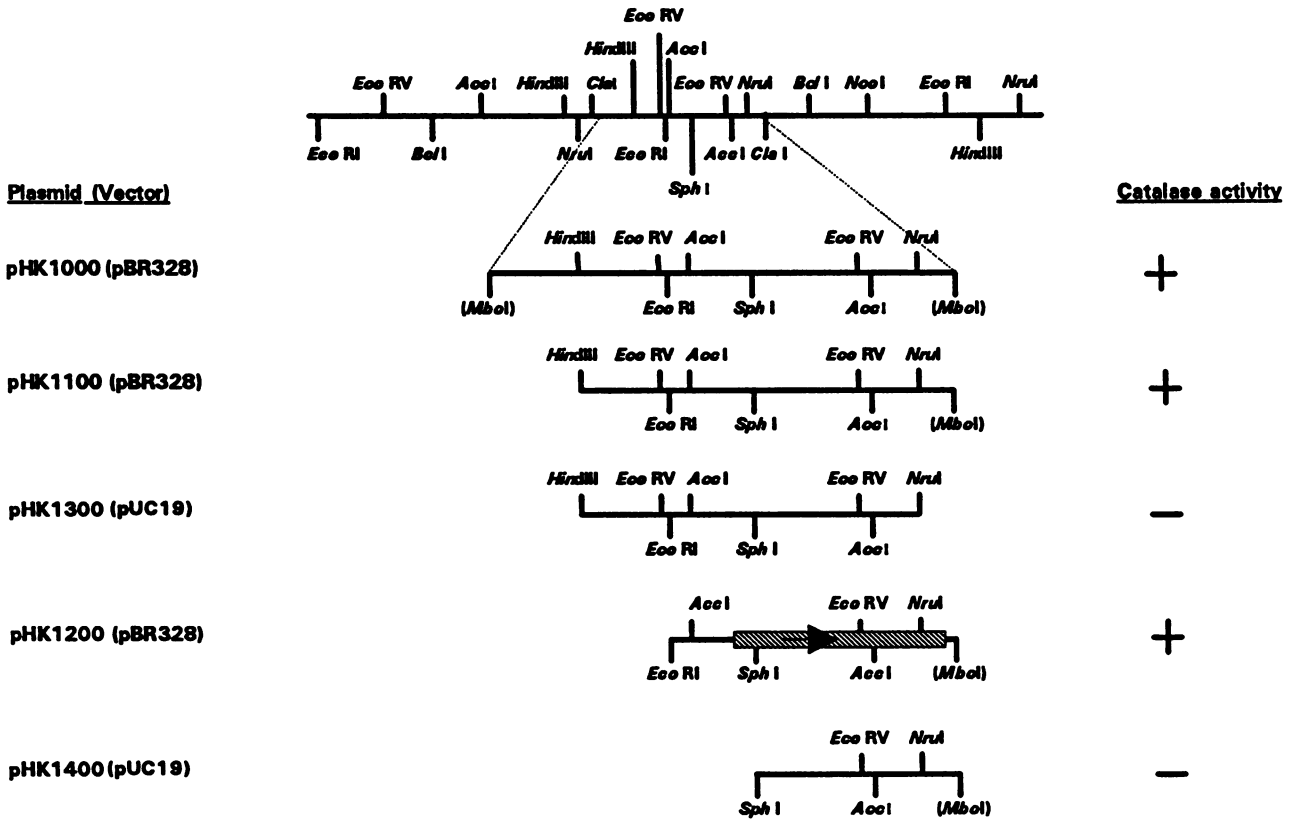

FIG. 2. Restriction map of *L. sake* LTH677 chromosomal DNA surrounding the catalase gene and deletion derivatives of pHK1000. pHK1100, *Hin*dIII fragment of pHK1000 religated; pHK1300, *Hin*dIII-*Nru*I fragment of pHK1000 cloned into pUC19; pHK1200, *Eco*RI fragment of pHK1000 religated; pHK1400, *Sph*I fragment of pHK1000 cloned into pUC19. The ability to complement the Kat$^-$ phenotype of *E. coli* UM2 is indicated by + and −. The bar in the insert of pHK1200 presents the position of the catalase structural gene as determined by nucleotide sequencing, the direction of transcription is indicated by an arrow.

chromosome of *L. sake* LTH677 provides evidence that it should be possible to reclone the catalase gene on single 4.5-kb *Cla*I, 9-kb *Bcl*I, or 7.6-kb *Hin*dIII fragments. However, all attempts to clone one of these fragments led to the formation of unstable plasmids.

Cloning the catalase gene in *L. casei*. To introduce the catalase gene into *L. casei* 102S, the gene was recloned into the *E. coli-Lactococcus* shuttle vector pGKV210, which replicates in lactobacilli, including *Lactobacillus curvatus* and *L. sake* (unpublished data). The *Hin*dIII-*Bam*HI fragment of pHK1150 containing the catalase gene and part of pUC19 was cloned into pGKV210, resulting in plasmid pHK1155 (Fig. 1). After amplification in *E. coli* UM2, plasmid pHK1155 was transformed into *L. casei* 102S by electroporation. All erythromycin-resistant transformants exhibited catalase activity on MRS plates containing hematin and harbored pHK1155, as confirmed by restriction analysis.

Homology of the catalase gene of *L. sake* LTH677 to catalase genes of other lactic acid bacteria. To investigate the homology of the catalase gene of *L. sake* LTH677 with catalase genes of other lactic acid bacteria, chromosomal DNAs of *Lactobacillus pentosus* DSM20314, *Pediococcus acidilactici* DSM20286, and *L. sake* LTH682, each of which possesses a heme-dependent catalase (35), were used in Southern hybridization experiments, with the *Acc*I fragment of the catalase gene used as a probe. The stringency was adjusted so that hybridizations of fragments having 60% homology

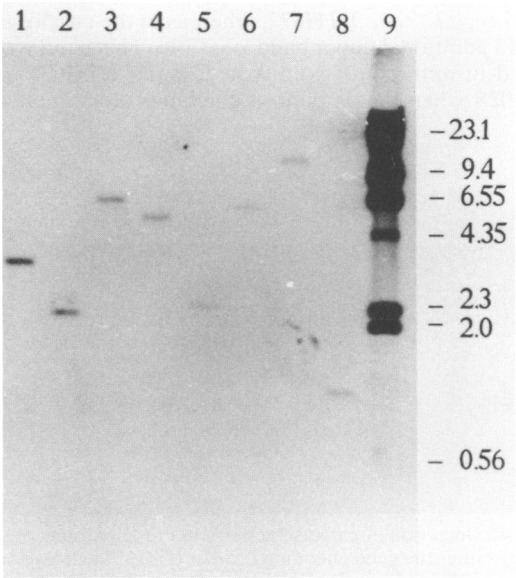

FIG. 3. Hybridization of part of the catalase gene to chromosomal DNA of *L. sake* strains. The probe consisted of the 1.7-kb *Acc*I fragment inside the catalase gene and labeled λ DNA. The chromosomal DNA of *L. sake* LTH677 (lanes 1 to 4) or *L. sake* LTH682 (lanes 5 to 8) was cut with *Bcl*I and *Eco*RI (lanes 1 and 5), *Cla*I and *Eco*RI (lanes 2 and 6), *Hin*dIII and *Eco*RI (lanes 3 and 7), or *Nco*I and *Eco*RI (lanes 4 and 8). Lane 9, λ molecular size markers (in kilobases).

TABLE 2. Catalase activities of different strains containing either *katS* or *katG*

Strain	Catalase activity [μmol of H_2O_2 × min^{-1} × (3×10^8 CFU)$^{-1}$]
L. sake LTH677 (wild type)	300–500
L. casei 102S(pHK1155)	250–400
L. casei 102S(pGKV210)	0
E. coli UM2(pHK1155)	300–500
E. coli UM2(pHK1150)	100–200
E. coli UM2(pGKV210)	0
E. coli UM2(pBT22)	400–500

could occur. Under these conditions, no signals were obtained with DNA of *L. pentosus* DSM20314 or *P. acidilactici* DSM20286. The hybridization patterns of *L. sake* LTH677 and LTH682 were different and are depicted in Fig. 3. No signal was detected by using pBT22, which contains the *E. coli katG* gene (encoding the catalase-hydroperoxidase HPI), as a probe (data not shown).

Expression of the catalase gene of *L. sake* LTH677 in *E. coli* UM2 and *L. casei* 102S. *L. casei* 102S(pHK1155) exhibited catalase activity when exogenous hematin was supplied. Without exogenous hematin, no catalase activity was detected. No dependence on exogenous hematin was detected in *E. coli* UM2. The catalase activities of the various strains are given in Table 2. Although the pUC19 derivative pHK1150 was present in high copy number, the catalase activity was not increased in transformants harboring this plasmid. All experiments were repeated at least three times. It was observed that, depending on the preparation, the cells exhibited variable activities in the range listed in Table 2.

Native-molecular-weight analysis. In Fig. 4, the result of the activity staining of a nondenaturing polyacrylamide gel is depicted. For *L. casei* 102S, the pattern is the same as it is for wild-type *L. sake* LTH677, whereas in the case of *E. coli* UM2, an additional upper band is visible. This band was also detected in extracts of wild-type *L. sake* LTH677 and *L. casei* 102S when the amount of soluble protein applied was

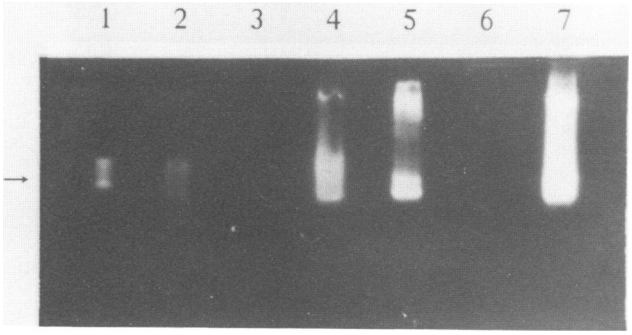

FIG. 4. Detection of catalase activity in crude extracts of strains harboring either the gene encoding *L. sake* LTH677 catalase or *katG* encoding HPI of *E. coli*. Soluble proteins were separated on nondenaturing 7.5% polyacrylamide gels of *L. sake* LTH677 (wild type) (lane 1), *L. casei* 102S(pHK1155) (lane 2), *L. casei* 102S (pGKV210) (lane 3), *E. coli* UM2(pHK1155) (lane 4), *E. coli* UM2 (pHK1150) (lane 5), *E. coli* UM2(pGKV210) (lane 6), and *E. coli* UM2(pBT22) (lane 7). The arrow indicates the position of the trimeric or dimeric form of the catalase; the upper band corresponds to the hexameric or tetrameric form (see text).

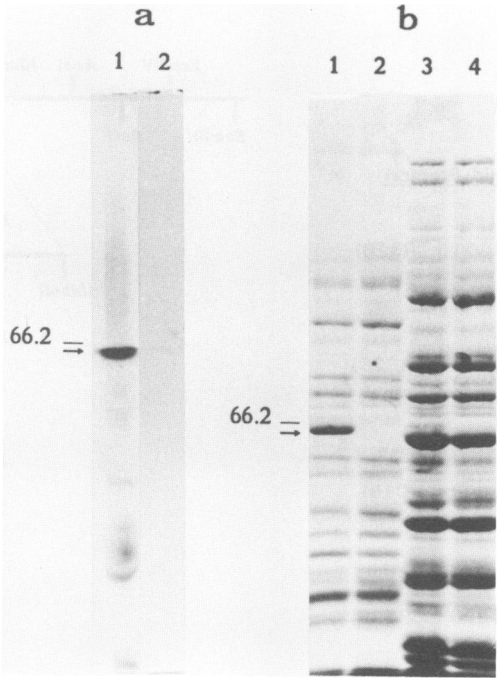

FIG. 5. (a) Subunit analysis of the *L. sake* LTH677 catalase on a SDS–7.5% polyacrylamide gel. Catalase proteins of *L. sake* LTH677 (wild type) (lane 1) and *L. casei* 102S(pHK1155) (lane 2) were obtained as described in Materials and Methods. The arrow indicates the position of putative catalase subunits of approximately 65,000 Da. (b) Total soluble proteins of strains harboring the gene encoding the catalase of *L. sake* LTH677. Lanes: 1, *E. coli* UM2 (pHK1155); 2, *E. coli* UM2(pGKV210); 3, *L. casei* 102S(pHK1155); 4, *L. casei* 102S(pGKV210). The arrow indicates the position of the additional 65,000-Da protein. In both panels, 66.2 indicates the molecular weight, in thousands, of a marker protein.

increased by a factor of 4. The molecular size of the native protein in each band was determined by the method of Hedrick and Smith (14). The molecular sizes of the proteins in the upper and lower band were approximately 340,000 and 180,000 Da, respectively. As a control, the molecular sizes of the upper and lower band caused by HPI in nondenaturing gels were determined to be 340,000 and 170,000 Da, respectively, confirming the findings of Loewen and Switala (18).

Protein analysis. For size determination of putative catalase subunits, total soluble proteins in the cell lysates were examined on a denaturing SDS–7.5% polyacrylamide gel. In *E. coli* UM2(pHK1155), an additional protein band with a molecular size of approximately 65,000 Da was detected in crude extracts (Fig. 5b). In *L. casei* 102S(pHK1155), an additional protein of the same size was detected in small amounts.

Subunit analysis. The analysis of proteins exhibiting catalase activity was performed as described in Materials and Methods. Figure 5a shows one major protein band, with the same size in lanes 1 and 2, enriched after two-step electrophoresis of *L. sake* LTH677 or *L. casei* 102S(pHK1155) catalase. These proteins are putative catalase subunits. Their molecular size was approximately 65,000 Da. This indicates that (i) the 65,000-Da proteins in Fig. 5b, lanes 1 and 3, are catalase subunits and (ii) the upper and lower bands in nondenaturing polyacrylamide gels are probably

```
   1  GCG TGT CTA AAT ACC ACT AAC CCC GAA AAA GAA CTG CCG ATA ACC TCG GCA GTT CTT TTT AGT AAC TTG TTG AGC AAG CTC TTC    84

                                                  *
  85  ATT GAC GGT GCC TGT TGA AGG TCC TAT AGT GAC CTA GGT AAG TTG CGC ACC ATC TTT TCA GTG TGT TCC ATG TTT TTA ACT ATT   168
        - 35                      - 10

 169  CTT AGG AGG TCA AAT ATT ATG ACA AAT CAA CTA ACG ACT AAC GAG GGG CAA CCG TGG GCG GAC AAT CAA CAA TTC GGC AAA CTG   252
   1       rbs               M   T   N   Q   L   T   T   N   E   G   Q   P   W   A   D   N   Q   Q   F   G   K   L    22

 253  CCG GCC AAC GCG GCC CCG TCC TTA ATC CAA GAT TAT CAA TTA CTC GAA AAA CTC GCC CAC TTT AAC CGC GAA CGC ATC CCT GAA   336
  23   P   A   N   A   A   P   S   L   I   Q   D   Y   Q   L   L   E   K   L   A   H   F   N   R   E   R   I   P   E    50

 337  CGG GTG GTG CAT GCC AAA GGC GCT GGC CTA AAG GCT ATT TCA AGG TTA CCA AGG ACA TTG AGC GCA TAT ACC AAA GCC GCT GTT   420
  51   R   V   V   H   A   K   G   A   G   L   K   A   I   S   R   L   P   R   T   L   S   A   Y   T   K   A   A   V    78

 421  TTC AGT GGC GTC GGC AAA AAA ACA CCG CTT ATC ACT CGT TTT TCT CAA GTC GCT GGT GAA GCC GGC TAT CCG ATA CAT ACC GCG   504
  79   F   S   G   V   G   K   K   T   P   L   I   T   R   F   S   Q   V   A   G   E   A   G   Y   P   I   H   T   A   106

 505  AGT GTC CGC GGT TTC GCC GTT AAA TTC TAT ACG GAA GAA GGC AAT TAC GAT ATT GTC GGC AAT AAC ACG CCG GTC TTC TTC GTC   588
 107   S   V   R   G   F   A   V   K   F   Y   T   E   E   G   N   Y   D   I   V   G   N   N   T   P   V   F   F   V   134

 589  AAT GAT CCA CTA AAA TTC CCC GAT TTC ATC CAC TCT CAA AAA CGT GAT CCC CGG ACA CAT GCC CGT AGC CAA GAT ATG CAA TGG   672
 135   N   D   P   L   K   F   P   D   F   I   H   S   Q   K   R   D   P   R   T   H   A   R   S   Q   D   M   Q   W   162

 673  GAT TTC TGG TCC CTG TCA CCC GAA TCT GTC CAC CAA GTC ACG ATT CTC ATG AGT GAT CGC GGG ATT CCT GCT AGT TAC CGG ATG   756
 163   D   F   W   S   L   S   P   E   S   V   H   Q   V   T   I   L   M   S   D   R   G   I   P   A   S   Y   R   M   190

 757  ATG CAC GGC TTT GGT AGC CAC ACC TTC AAA TGG GTT AAC GCA CAA GGT GAA CAA TTC TGG GTT ATA TTC CAT TTC AAG ACG AAC   840
 191   M   H   G   F   G   S   H   T   F   K   W   V   N   A   Q   G   E   Q   F   W   V   I   F   H   F   K   T   N   218

 841  CAA GGT ATT CAC CAA TCT CAG CAA CGA ACT CGG CCG ATG AAC TCG CTG GTA AGG ATA CTG ATT ACC TTC AAA ATG ATT TAT TCG   924
 219   Q   G   I   H   Q   S   Q   Q   R   T   R   P   M   N   S   L   V   R   I   L   I   T   F   K   M   I   Y   S   246

 925  ACG CAA TTG AAA CCG CGA TTA TCA AGT TGG ACG GTG TGC CGT CCA ACT CGT CCT TAT GAA GAT GGC TTG AAT TAT CTC CCA AGG  1008
 247   T   Q   L   K   P   R   L   S   S   W   T   V   C   R   P   T   R   P   Y   E   D   G   L   N   Y   L   P   R   274

1009  ATA TTT TTG ATG TTA CTA AAG GTT ATT TCA CAA AAG GAT TAT CCA TTA ATC GAA ATC GGT CAA ATG GTC CTC GAT GAA AAT CCA  1092
 275   I   F   L   M   L   L   K   V   I   S   Q   K   D   Y   P   L   I   E   I   G   Q   M   V   L   D   E   N   P   302

1093  ACG AAT AAC TTC GAA GAT ATC CAA GAA CTG GCC TTC TCA CCG GCT AAC TTA GTC CCT GGG ATT GAA GCA TCA CCC GAC AAA TTA  1176
 303   T   N   N   F   E   D   I   Q   E   L   A   F   S   P   A   N   L   V   P   G   I   E   A   S   P   D   K   L   330

1177  CTT CAA GGT CGA CTA TTT GGC TAT AAG GAT GCT GAA CGT TAC CGG CTT GGT GCC AAC TAC GAG CAA CTC CCT GTC AAC CGA CCA  1260
 331   L   Q   G   R   L   F   G   Y   K   D   A   E   R   Y   R   L   G   A   N   Y   E   Q   L   P   V   N   R   P   358

1261  AAA GTC CCC GTT CAT AAT TAC GAA CGT GAC GGT GCG ATG GCC CAA AAT CAA GCA ACT GGC GTT AAC TAC GAA CCC AAC AGT CAA  1344
 359   K   V   P   V   H   N   Y   E   R   D   G   A   M   A   Q   N   Q   A   T   G   V   N   Y   E   P   N   S   Q   386

1345  GAT GGA CCC ACT GAA GTC CCA GCA GCT AAG ATT CAT GGC GAT CAA CTC TCT GGT ACA ACT GGC AAC TTC TCT GCC GAT CCC GAT  1428
 387   D   G   P   T   E   V   P   A   A   K   I   H   G   D   Q   L   S   G   T   T   G   N   F   S   A   D   P   D   414

1429  TAT TAC TCA GCA GCT GGC AAA CTT TAC CGG TTA TTA TCA GCC GAT GAA CAA ACC CGC TTA ATC GAA AAT ATT CGC ATG AAT CTT  1512
 415   Y   Y   S   A   A   G   K   L   Y   R   L   L   S   A   D   E   Q   T   R   L   I   E   N   I   R   M   N   L   442

1513  GGT CAA GTG ACT AAA CCA GAA ATT CAA ATT CGC GAA GTT AAA CAA TTT TAC CAA GCT GAT CCA GAA TAT GGT CGG CGC GTC GCA  1596
 443   G   Q   V   T   K   P   E   I   Q   I   R   E   V   K   Q   F   Y   Q   A   D   P   E   Y   G   R   R   V   A   470

1597  ACC AGC GTT AAA CTT AGA TTT AGC TCA GTT TGA ATA ATC ACT AAC ACG AAA AAA TAG GTG GCC CCA GTT TGG GAC ACC TAT TTT  1680
 471   T   S   V   K   L   R   F   S   S   V                   ---------- --->       <--- ----------                  480

1681  TTA TTC GTT ATC TTT TTT CCC TGT CTC TTG TTT TGC CGC CAC TCT TTT TTC GGT TAA TAA TCA GAA ACG TCA TCT TGA TTC GTA  1764
        -  ------

1765  ACG TTA AGC AGT CAA TTA TGC                                                                                    1785
```

FIG. 6. Nucleotide sequence of the *L. sake* LTH677 catalase structural gene and deduced amino acid sequence of the catalase. Promoter sequences homologous to the −35 and −10 regions of *E. coli* are underlined, as is the putative ribosome binding site (rbs); the transcription start site is marked by an asterisk. An inverted repeat capable of forming a stem-loop structure is marked by dashed arrows.

hexameric and trimeric aggregates, respectively, of the 65,000-Da catalase subunit.

Nucleotide sequence of the catalase gene. Nucleotide sequencing of the insert DNA of pHK1200 revealed one open reading frame whose sequence comprised 1,440 bp encoding a protein of 480 amino acids with a calculated molecular size of 54,504 Da (Fig. 6). The amino acid sequence of the additional protein produced by *E. coli* UM2(pHK1155) determined as described in Materials and Methods was consistent with the amino acid sequence predicted from the nucleotide sequence (Fig. 7). As this protein also corresponded to the protein observed during subunit analysis of the active catalase, it can be concluded that the catalase structural gene has been cloned. As it is the first catalase gene isolated from a *Lactobacillus* strain, it is designated *katA*.

Flanking nucleotide sequences. A −10 region similar to the

a)	ATG	ACA	AAT	CAA	CTA	ACG	ACT	AAC	GAG	GGG
b)	Met	Thr	Asn	Gln	Leu	Thr	Thr	Asn	Glu	Gly
c)	*	*	Asn	Gln	Leu	Thr	*	Asn	Glu	Gly

*: not determinable

FIG. 7. Comparison between the N-terminal amino acid sequence of the catalase subunit deduced from the DNA sequence (b), starting with the first ATG codon of the open reading frame (a), and the amino acid sequence determined by protein microsequencing (c).

E. coli consensus sequence was detected upstream of the start ATG codon (TATAGT in *L. sake* LTH677 DNA and TATAAT in *E. coli* DNA [13]). A putative −35 region similar to the corresponding region in *E. coli* (TTGACG in *L. sake* LTH677 DNA and TTGAAG in *E. coli* DNA [13]) was also present. The distance between the two regions was 17 bp. A putative ribosome binding site, which was identical to the corresponding region in *E. coli* (29) (AGGAGG in both organisms), was found 9 bp upstream of the ATG start codon.

Downstream of the TGA stop codon, a sequence resembling the structure of an *E. coli* ρ-independent transcriptional terminator was detected. The RNA transcribed from this region would form a stem-loop structure followed by a stretch of U residues (Fig. 6). The calculated free energy of formation for this structure would be −117.3 kJ, which is within the range typically observed for ρ-independent terminators in *E. coli* (23).

DISCUSSION

Catalase activity in lactobacilli is a rare property which can prevent flavor and color defects in fermented foods. Strains of lactobacilli exhibiting this rare property have been summarized by Hammes et al. (11). The understanding of the regulation of the catalase gene expression and of properties of the catalase enzyme not only will allow comparison with catalases and hydroperoxidases of other organisms but will be helpful in determining optimized technical conditions for flavor improvement of fermented meat products. In addition, the cloned *katA* gene can be transferred to other starter organisms as a new, desirable trait and hence can directly enhance their performance.

Attempts to clone fragments larger than 6 kb in pBR328 led to the formation of structurally unstable plasmids, resulting in excision and deletion formation. Furthermore, reclonning of *katA* on known *Cla*I, *Bcl*I, or *Hin*dIII chromosomal DNA fragments failed. This may be due to pseudopromoter activity of cloned AT-rich regions of *Lactobacillus* DNA in *E. coli* which interfere with plasmid replication (4). On the other hand, DNA rearrangements during shotgun cloning or recombination events in *E. coli* UM2 were excluded by (i) using the *rec* strain *E. coli* BHB2600 as an intermediate host for the primary construction and amplification of the gene library and (ii) analysis of the original chromosomal arrangement of the cloned fragment in pHK1000 by Southern hybridization.

In pHK1155 and pHK1250, both possible orientations of *katA* are flanked by genes or parts of genes of the vector which are transcribed downstream of the cloning site, thus excluding translational coupling. The expression of the catalase in *L. casei* 102S gives support to the claim that the *Lactobacillus* promoter had been cloned. In addition, the expression in *E. coli* was probably directed by the regulatory nucleotide sequences present on the cloned *L. sake* LTH677 DNA, because the catalase activity was independent of the orientation of the insert and recognition sequences homologous to the *E. coli* consensus sequences were present upstream of the coding region.

The catalase subunit size determined by SDS-polyacrylamide gel electrophoresis was approximately 10,000 Da larger than that deduced from the DNA sequence. This may be caused by the acidic character of the protein (32) (21.9% acidic amino acids versus 13.9% basic amino acids); the catalase subunits migrated more slowly than the standard proteins.

The subunit size of the *L. sake* LTH677 catalase corresponds well with the sizes of the catalases commonly isolated from animals, plants, and microorganisms (22). However, these enzymes are composed of four subunits of equal size with a combined molecular size in the range of 225,000 to 270,000 Da. The native molecular sizes of KatA determined by native polyacrylamide gel electrophoresis were 180,000 and 340,000 Da, corresponding to a trimeric and a hexameric structure, respectively. The conditions in the gel may cause a partial breakdown of KatA, with the trimeric form being the smallest active form of the enzyme. Loewen and Switala (18) observed the same phenomenon for both catalases of *E. coli*, HPI and HPII. The unusual hexameric structure of KatA differs from most other catalases with tetrameric structure. So far, the only known catalases with a hexameric structure are HPII from *E. coli* (18) and catalase-1 from *Bacillus subtilis* (19). Whereas the former has a subunit size of approximately 84,000 Da, the latter consists of 65,000-Da subunits. Thus, with regard to subunit size and hexameric structure, KatA strongly resembles catalase-1 from *B. subtilis*.

Within the lactic acid bacteria, there exist at least two different catalases, since *katA* hybridized only to DNA of *L. sake* LTH682, whereas the DNA of *L. pentosus* DSM20314 and *P. acidilactici* DSM20286 did not hybridize with *katA*. No homology was also detected with the catalase-peroxidase HPI of *E. coli* under the conditions used.

Catalase-positive transformants of *E. coli* UM2 survived for at least 2 min after the colonies were flooded with hydrogen peroxide, thus allowing the recovery of positive clones. This also applied to catalase-positive lactobacilli, indicating the suitability of *katA* as a nonantibiotic marker gene for the construction of food grade vectors, as most lactobacilli are Kat^-. Although there is no positive selection for catalase-positive clones, the easy detection and recovery of Kat^+ transformants allow the screening of many clones in a short time.

katA provides all genetic information required for Kat^- lactobacilli to form an active catalase if a heme source is provided. Since fermenting substrates of plant and animal origin usually contain sufficient porphyrinoids to ensure catalase activity (35), a practical use of *katA* to improve starter strains is possible. Thus, the effect of catalase on sensory quality of fermented foods can be investigated by means of Kat^+ derivatives of lactobacilli which are adapted to these specific environments.

ACKNOWLEDGMENTS

We are indebted to F. Goes (Institut für Lebensmitteltechnologie) for performing the amino acid sequencing and G. Venema (University of Groningen) for critical reading of the manuscript. We thank J.

Kok (University of Groningen) for providing plasmid pGKV210 and P. C. Loewen for plasmid pBT22 and *E. coli* UM2.

This work was supported by Bundesministerium für Forschung und Technologie grant 0319280A.

The authors are responsible for the contents of the publication.

REFERENCES

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46:**549–552.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Chassy, B. M., and J. L. Flickinger.** 1987. Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol. Lett. **44:**173–177.
4. **Chen, J.-D., and D. A. Morrison.** 1987. Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by strong transcriptional terminators. Gene **55:**179–187.
5. **Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. **140:**532–537.
6. **Davies, R., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 140–141. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A method for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130–135.
8. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
9. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. Recovery of DNA fragments from agarose gels using DEAE paper. Anal. Biochem. **112:**295.
10. **Gaier, W., R. F. Vogel, and W. P. Hammes.** 1990. Genetic transformation of intact cells of *Lactobacillus curvatus* Lc2-c and *Lact. sake* Ls2 by electroporation. Lett. Appl. Microbiol. **11:**81–83.
11. **Hammes, W. P., A. Bantleon, and S. Min.** 1990. Lactic acid bacteria in meat fermentation. FEMS Microbiol. Rev. **87:**165–174.
12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
13. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
14. **Hedrick, J. I., and A. J. Smith.** 1964. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126:**155–164.
15. **Hohn, B.** 1979. *In vitro* packaging of λ and cosmid DNA. Methods Enzymol. **68:**299–309.
16. **Knauf, H. J., R. F. Vogel, and W. P. Hammes.** 1989. Introduction of the transposon Tn*919* into *Lactobacillus curvatus* Lc2-c. FEMS Microbiol. Lett. **65:**101–104.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Loewen, P. C., and J. Switala.** 1986. Purification and characterization of catalase HPII from *Escherichia coli* K12. Biochem. Cell Biol. **64:**638–646.
19. **Loewen, P. C., and J. Switala.** 1987. Purification and characterization of catalase-1 from *Bacillus subtilis*. Biochem. Cell Biol. **65:**939–947.
20. **Loewen, P. C., B. L. Triggs, C. S. George, and B. E. Hrabarchuk.** 1985. Genetic mapping of *katG*, a locus that affects synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in *Escherichia coli*. J. Bacteriol. **162:**661–667.
21. **Moore, W. E. C., D. E. Hash, L. V. Holdeman, and E. P. Cato.** 1980. Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. Appl. Environ. Microbiol. **39:**900–907.
22. **Nadler, V., I. Goldberg, and A. Hochman.** 1986. Comparative study of bacterial catalases. Biochim. Biophys. Acta **882:**234–241.
23. **Platt, T.** 1986. Transcription termination and regulation of gene expression. Annu. Rev. Biochem. **55:**339–372.
24. **Rozier, J.** 1971. Die Rolle der Katalase-Aktivität des Fleisches bei der Rohwurst-Fabrikation. Fleischwirtschaft **7:**1063–1066.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
27. **Sinha, A. K.** 1972. Colorimetric assay of catalase. Anal. Biochem. **47:**389–394.
28. **Soberon, X., L. Covarrubias, and F. Bolivar.** 1980. Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene **9:**287–306.
29. **Stromo, G. D., T. D. Schneider, and L. M. Gold.** 1982. Characterization of translational initiation sites in *E. coli*. Nucleic Acids Res. **10:**2971–2995.
30. **Triggs-Raine, B. L., and P. C. Loewen.** 1987. Physical characterization of *katG*, encoding catalase HPI of *Escherichia coli*. Gene **52:**121–128.
31. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**540–542.
32. **von Ossowski, I., M. R. Mulvey, P. A. Leco, A. Borys, and P. C. Loewen.** 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. J. Bacteriol. **173:**514–520.
33. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextrane sulfate. Proc. Natl. Acad. Sci. USA **76:**3683–3687.
34. **Wolf, G., and W. P. Hammes.** 1988. Effect of hematin on the activities of nitrite reductase and catalase in lactobacilli. Arch. Microbiol. **149:**220–224.
35. **Wolf, G., A. Strahl, J. Meisel, and W. P. Hammes.** 1991. Heme-dependent catalase activity of lactobacilli. Int. J. Food Microbiol. **12:**133–140.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.